STUDIES ON ENZYME RADIOSENSITIVITY

I. THE EFFECT OF BICARBONATE ON LIVER ALCOHOL DEHYDROGENASE

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(Received January 7th, 1960)

SUMMARY

When dilute aqueous solutions of liver alcohol dehydrogenase were exposed to X-irradiation, there was much more inactivation in the presence of bicarbonate buffer than in phosphate buffer or in distilled water. The effect was not due to the action of any stable intermediates produced by irradiation of bicarbonate buffer, nor does it seem likely that the enzyme is converted by irradiation to some long-lived metastable state whose destruction is precipitated by the presence of bicarbonate. Enzyme and bicarbonate must be present simultaneously for the effect to be observed. Several other enzymes have been investigated in this regard, and possible mechanisms discussed.

INTRODUCTION

A wide variety of data are available in the literature concerning the inactivation of purified enzymes by ionizing radiations. DALE[1-4] and BARRON[5,6] have laid important groundwork in this area. Other such studies have been carried out by MCDONALD[7-9] with trypsin and chymotrypsin, by SETLOW AND DOYLE[10], BONET-MAURY AND PATTI[11] and SUTTON[12] with catalase, by ANDERSON[13] with pepsin, by BABIN[14] with papain, and by ROBINSON, PHILLIPS AND DILG[15] with a variety of enzymes. Uniformly, these workers have reported that added solutes either protected the enzyme from irradiation damage or else had no effect, except in the case of dissolved oxygen which increased the radiosensitivity of several sulfhydryl enzymes[16]. It was therefore of great interest in the present study to observe the striking enhancement of the radiosensitivity of liver alcohol dehydrogenase in the presence of bicarbonate buffers.

EXPERIMENTAL

Enzyme

Crystalline horse liver alcohol dehydrogenase (lot No. 5521) was obtained from the Worthington Biochemical Corporation. When assayed by the method of BONNICHSEN *et al.*[17] this was found to be about 20% active enzyme. For subse-

quent assays DPN reduction was followed between 0.5 and 1.5 min rather than for 3 min as in the method of BONNICHSEN, and Tris buffer was used rather than glycine buffer.

Irradiation

250 kV X-rays were used. The enzyme samples were dissolved in 1 ml of solution and exposed in 5 ml Pyrex glass beakers using a Plexiglas holder for reproducible positioning in the X-ray beam. For irradiation in gases other than air a sealed holder was used. This was evacuated for 10 min with a water aspirator, flushed for 5 min with nitrogen or helium and the evacuation and flushing repeated before sealing off the system containing the inert gas at atmospheric pressure. Ferrous-ferric dosimetry[18] was used routinely and (since the dosimeter yield is dependent on oxygen saturation) indicated thorough removal of oxygen by this procedure.

The enzyme concentration was 100 μg protein/ml of solution. Following the irradiation period, 0.1 ml aliquots were withdrawn from each sample for assay. Controls were shielded with lead and kept in the X-ray room during exposure of the other samples.

Comparison of buffers

Enzyme was irradiated at a dose of 7–12 Krep in phosphate and bicarbonate buffers at various pH values but all at 0.1 *M* concentration. The inactivation date are plotted in Fig. 1. In the range between pH 8 and 9, where it was possible to overlap these buffers, the difference between them is apparent. This difference was clearly not a pH effect, but, rather, was dependent on the specific anions. Other buffers could not conveniently be used in this range because they strongly protected the enzyme against X-irradiation. For example, with 0.1 *M* borate buffer an X-ray dose of 20 Krep was required to achieve inactivation similar to 10 Krep with phosphate buffer. Some data with borate are given in Fig. 2 and indicate increased protection at decreased pH values. This may indicate that boric acid is more effective as a protector than is borate anion. These observations, however, have not been studied further.

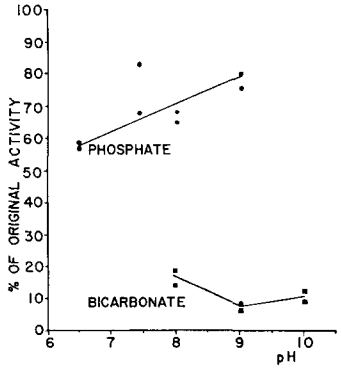

Fig. 1. Per cent of activity of liver alcohol dehydrogenase remaining after 12 Krep X-irradiation in 0.1 *M* phosphate or bicarbonate at various pH values. Each sample had 100 μg protein dissolved in 1 ml of buffer.

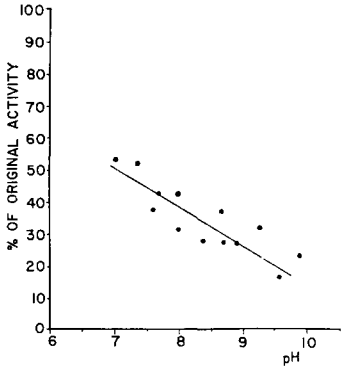

Fig. 2. Per cent of activity of liver alcohol dehydrogenase remaining after 30 Krep X-irradiation in 0.1 *M* sodium borate buffer at various pH values. Each sample had 100 μg protein dissolved in 1 ml of buffer.

Assay by the reverse reaction

A comparison between phosphate and bicarbonate buffers was also made by using the reverse reaction:

$$\text{acetaldehyde} + \text{DPNH} \longrightarrow \text{ethanol} + \text{DPN}$$

to assay enzyme activity. For this method of assay the following additions were made to a 1 cm Beckman cuvette: 0.50 ml Tris buffer 0.5 *M*, pH 8.5, 2.25 ml water, 0.05 ml 0.005 *M* DPNH, 0.10 ml 10 % v/v acetaldehyde.

Enzyme was added (0.1 ml) and the change in absorption between 1 and 2 min read at 340 mμ. Because of the rapidity of this reverse reaction the enzyme samples were diluted by adding 2 ml of water to the 1 ml samples before withdrawing 0.1-ml aliquots. The results of this assay were entirely comparable with the data obtained from the forward reaction (Fig. 1) so that the bicarbonate sensitization applies to the inactivation of the catalytic activity regardless of the direction of the reaction catalyzed.

Inertness of phosphate

The data of Fig. 1 without further evidence could be interpreted as resulting either from protection by phosphate or sensitization by bicarbonate. It was found that at pH 6.5 irradiation in 0.1 *M* phosphate gave just as much inactivation as irradiation in distilled water at this pH. The results of more conclusive experiments are given in Fig. 3 and Table I. Fig. 3 shows that as the concentration of bicarbonate buffer was reduced to zero while keeping the pH constant at 9, the amount of enzyme inactivated approached the value observed in phosphate buffer. Varying the phosphate concentration had a negligible effect on enzyme radiosensitivity. If phosphate were protective and bicarbonate inert, it would be expected that the curve for the latter would be a straight line cutting the ordinate at the same point as the curve for phosphate extrapolated to zero phosphate concentration. The activity for phosphate would then rise with increasing phosphate concentration.

Further corroboration for the inertness of phosphate is shown in Table I. Here, activity is given for the two buffers separately and mixed. All activities were the

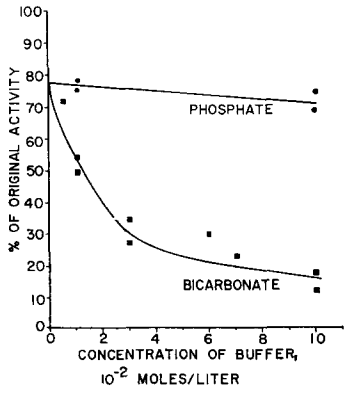

Fig. 3. Inactivation of liver alcohol dehydrogenase in phosphate and bicarbonate buffers at different concentrations but all at pH 9. X-ray dose: 12 Krep. Each sample had 100 μg protein in 1 ml of buffer.

TABLE I

INACTIVATION OF LIVER ALCOHOL DEHYDROGENASE BY X-IRRADIATION IN PHOSPHATE AND BICARBONATE BUFFER SEPARATELY AND MIXED

X-ray dose 12 Krep. Enzyme concentration 100 μg/ml. Activity values represent the O.D. change in 1 min at 340 mμ.

	Buffer used (0.1 M, pH 9)	*Activity*
X-rayed Samples	Bicarbonate	0.015
		0.008
	Phosphate	0.049
		0.043
	Mixed bicarbonate + phosphate	0.015
		0.010
Non-X-rayed Controls	Bicarbonate	0.055
		0.056
	Phosphate	0.056
		0.061
	Mixed bicarbonate + phosphate	0.056
		0.057

same in non-irradiated samples, whereas in the mixed buffer there was the same amount of inactivation as in bicarbonate alone, indicating no protection by the phosphate; or, stated in another way, the bicarbonate produced its sensitization effect even in the presence of phosphate.

Effect of bicarbonate on non-irradiated enzyme

The data presented above have shown that, at the concentration of enzyme and buffer normally used, bicarbonate had no inhibitory effect on the enzyme. The possibility remained that in a partially radiation-inactivated (*i.e.* more dilute) enzyme solution, inhibition by bicarbonate could produce an effect which would be interpreted as enhancement of radiosensitivity. To test this possibility several dilutions of enzyme were prepared in 0.1 *M*, pH 9.0 bicarbonate and phosphate buffers. These were assayed immediately and after a period of time. The results are shown in Table II. Even with the highest dilution of enzyme used there was no indication of significantly lower activity in bicarbonate than in phosphate buffer.

TABLE II

ACTIVITY OF LIVER ALCOHOL DEHYDROGENASE DILUTED IN BICARBONATE AND PHOSPHATE BUFFERS

Values represent O.D. change in 1 min at 340 mμ.

Enzyme concentration, μg/ml	*Buffer used*	*Activity immediately*	*Activity after 30 min*
100	Phosphate	0.016	0.013
100	Bicarbonate	0.021	0.013
75	Phosphate	0.034	0.037
75	Bicarbonate	0.035	0.034
50	Phosphate	0.044	0.049
50	Bicarbonate	0.043	0.042
25	Phosphate	0.061	0.062
25	Bicarbonate	0.056	0.053

Irradiation of enzyme and bicarbonate separately

To determine whether stable products formed on irradiation of bicarbonate solutions were responsible for inactivating the enzyme, solutions of 0.1 *M*, pH 9.0 phosphate and bicarbonate buffers were irradiated and then used for dilution of the enzyme in the same way as non-irradiated buffer solutions. Results of such an assay are given in Table III. There was no significant inactivation of enzyme by either of the irradiated solutions.

TABLE III

ADDITION OF IRRADIATED AND NON-IRRADIATED BUFFERS (0.1 *M*, pH 9) TO NON-IRRADIATED ENZYME

Final enzyme concentration 100 μg/ml. Buffer (0.9 ml) irradiated with 12 Krep and added immediately to 0.1 ml concentrated enzyme. Activity values represent change in O.D. in 1 min at 340 mμ.

	Buffer used	*Activity*
Irradiated Buffer	Phosphate	0.056
	Phosphate	0.064
	Bicarbonate	0.051
	Bicarbonate	0.053
Non-irradiated Buffer	Phosphate	0.059
	Phosphate	0.059
	Bicarbonate	0.056
	Bicarbonate	0.065
Enzyme irradiated in bicarbonate Buffer	—	0.010
		0.006

To see whether the enzyme might somehow be "weakened" by irradiation and thereby made susceptible to inactivation by bicarbonate, solutions of enzymes were irradiated in phosphate buffer and immediately mixed with bicarbonate buffer to give a final solution of pH 9.0 and 0.1 *M* in phosphate and bicarbonate. This was then assayed as usual, and typical results are given in Table IV. There was no indication that the suggested mechanism was operative.

TABLE IV

The enzyme was irradiated in phosphate buffer, pH 9, 0.125 *M*, at 12 Krep and then mixed with bicarbonate buffer or water to give final concentration of 0.1 *M* phosphate and bicarbonate before assaying. Enzyme concentration 100 μg/ml. Activity figures represent O.D. change in 1 min at 340 mμ.

	Diluted with water	*Diluted with bicarbonate*
Enzyme in phosphate, irradiated	0.045	0.050
	0.041	0.046
Enzyme in phosphate, control	0.060	0.060
	0.066	0.060
Enzyme irradiated in mixed buffer	0.024	—
	0.018	—

The oxygen effect

In an attempt to gain more insight as to possible mechanisms of the bicarbonate effect, enzyme solutions in phosphate and bicarbonate buffers (0.1 *M*, pH 9.0) were irradiated in atmospheres of nitrogen and helium for comparison with results obtained in air. Results of such experiments are given in Fig. 4. At the X-ray dose used, enzyme dissolved in phosphate buffer was negligibly inactivated whether in air or in an inert atmosphere. Enzyme dissolved in bicarbonate buffer, however, showed considerably more inactivation in the presence of oxygen; but, in any case, the increasing sensitivity of enzyme with increasing bicarbonate concentration was maintained regardless of the atmosphere used. Fig. 4 also shows that there was a very gradual loss of enzyme activity after irradiation whether in air or in nitrogen. Since this delayed inactivation appeared not to be accelerated when irradiation was carried out in air, it is presumably a simple thermal process and not the result of inactivation by hydrogen peroxide in the solution.

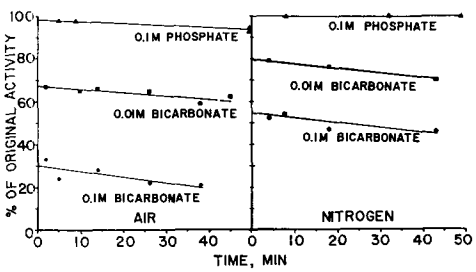

Fig. 4. A comparison of enzyme inactivation in nitrogen and air with three different buffer systems. The time coordinate represents time interval after the end of the X-ray period. X-ray dose: 7 Krep. Enzyme concentration, 100 μg/ml.

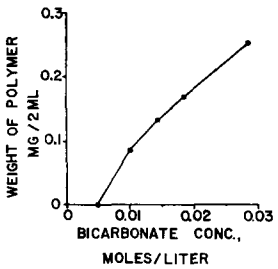

Fig. 5. Polymethylmethacrylate formed *vs.* concentration of bicarbonate in pH 9 aqueous solution. 2 ml of 0.1 *M* monomer irradiated with 35 Krep in a helium atmosphere.

A methacrylate model system

Since DAINTON[19], and others have shown that the polymerization of various acrylic monomers in aqueous solution is catalyzed by radiation-produced hydroxyl radicals, we have investigated the effect of bicarbonate on such a system to see whether it would increase the amount or speed of polymerization.

Methyl methacrylate was dissolved in deaerated water with various concentrations of bicarbonate buffer at pH 9 to give a 0.1 *M* solution of monomer. 2-ml samples were irradiated in a helium atmosphere at a dose of approximately 35 Kreps. Coagulated polymer was then separated by centrifuging, washed several times with water and determined quantitatively using a colorimetric, chromic acid oxidation method. Results of a typical experiment are plotted in Fig. 5. Polymer formation was definitely increased with increasing bicarbonate concentrations, although this was observed over a much narrower range of concentration than was found for the enzyme sensitization.

Other enzymes

Preliminary experiments have been carried out to see whether the bicarbonate sensitization effect applies to other enzymes as well as to liver alcohol dehydrogenase. With yeast alcohol dehydrogenase there was an apparent sensitization if samples

were assayed several minutes after irradiation. This, however, was found to be an artifact which resulted from the fact that dilute solutions of yeast alcohol dehydrogenase were more rapidly inactivated in bicarbonate than in phosphate buffer, regardless of irradiation. Other enzymes which were examined with negative results were yeast hexokinase, muscle aldolase, and muscle lactic dehydrogenase.

DISCUSSION

The data presented leave no doubt as to the validity of the bicarbonate sensitization effect. Clearly, liver alcohol dehydrogenase is much more sensitive to the action of X-irradiation in the presence of bicarbonate buffer than in its absence.

In general terms, three possible mechanisms can be adduced to explain the data: (a) Irradiation of bicarbonate solutions produces some highly reactive and unstable intermediate which is especially effective in attacking the enzyme. (b) When dissolved in bicarbonate buffer, the enzyme assumes a configuration which renders it particularly susceptible to attack by the free radicals produced in solution. These free radicals, however, are no different qualitatively or quantitatively from those produced by irradiation of phosphate buffers. (c) In bicarbonate buffer the same free radicals attack the enzyme as in phosphate buffer, but more of them are produced.

At present there is little basis for choosing a mechanism. GARRISON *et al.*[20], have shown that high energy alpha bombardment of carbonic acid solutions caused some reduction with the formation of small quantities of formate, formaldehyde and oxalate. These compounds, however, are all sufficiently stable so that if they were responsible for inactivating the enzyme, addition of irradiated buffer solution to the non-irradiated enzyme should have the same result as irradiation of the two simultaneously. In addition, judging from the data of GARRISON *et al.*, the concentrations of these substances which would have been formed in our system are vanishingly small.

If mechanism (a) applies, the required intermediate must be unstable and most likely a free radical. Peroxycarbonates are known to be produced by the reaction of hydrogen peroxide with alkali carbonates. These are strong oxidizing and reducing agents which presumably could be formed by irradiation of carbonate solutions in the presence of oxygen. However, they are not unstable enough to account for our data; and, more important, they presumably could not be formed in the absence of oxygen, where the bicarbonate sensitization was still observed.

Concerning mechanism (b), likewise, little can be said. However, the fact that bicarbonate strongly inhibits yeast alcohol dehydrogenase irrespective of irradiation may be taken as suggesting that it does have some effect on enzyme protein. Clarification of this inhibitory effect with the yeast enzyme might be helpful in understanding the mechanism of bicarbonate sensitization of the liver alcohol dehydrogenase.

At the present, a combination of mechanisms (b) and (c) seems best to explain the data. While water is decomposed into free radicals by radiation as follows:

$$H_2O \longrightarrow \cdot H + \cdot OH$$

the reverse reaction also readily occurs to limit the amount of hydroxyl radical present if nothing is available for the hydrogen to react with. As shown by the work

of GARRISON *et al.*[20] irradiated bicarbonate forms oxalate presumably as follows:

$$HCO_3^- \longrightarrow \cdot COO^- + \cdot OH$$

$$2 \cdot COO^- \longrightarrow \begin{matrix} COO^- \\ | \\ COO^- \end{matrix}$$

so that as the carboxyl radicals combine with each other to form oxalate, the hydroxyl radical is left without a partner and could reach greater concentrations than in the irradiation of pure water.

The methacrylate polymerization reaction, known to be catalyzed by hydroxyl radicals confirms the above hypothesis since polymerization was increased by increasing the bicarbonate concentration.

An increased concentration of hydroxyl radicals is alone insufficient to account for the bicarbonate effect with liver alcohol dehydrogenase, however, since other enzymes which are also inactivated by hydroxyl radicals were not found to be sensitized by bicarbonate. It seems reasonable to invoke mechanism (b) to explain this difference and to suggest that with this particular enzyme the bicarbonate may be more closely associated with the protein than with the other enzymes tested so that not only is the concentration of hydroxyl radicals increased but it is increased in the immediate vicinity of the enzyme groups to be inactivated. Carbon dioxide is known to form more or less tight complexes with the free amino groups of certain proteins[21], and such complexes might play a role here. At least their existence shows that bicarbonate may be closely associated with enzyme protein.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the U.S. Public Health Service and the Atomic Energy Commission.

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